

STIC-ILL

V. NO 2/10

From: Holleran, Anne
Sent: Tuesday, February 10, 2004 3:19 PM
To: STIC-ILL
Subject: refs. for 08/070,099

48/868

Please send copies of the following papers:

1. Allen J. Biol. Chem. (1973) 248: 3660-3669
2. Katz J. Clin. Invest (1974) 53: 1274-1283
3. Grasbeck Lancet (1982) I: 1330-1332

Anne Holleran
AU: 1642
Tel: (571) 272-0833
RM: Remsen, 3A14

mailbox: Remsen, 3C18

13057905

Isolation and Characterization of an Abnormal Human Intrinsic Factor

MAX KATZ, CAROL S. MEHLMAN, and ROBERT H. ALLEN

From the Division of Hematology, Royal Victoria Hospital and the Department of Experimental Medicine, McGill University, Montreal, Canada and the Division of Hematology-Oncology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT A patient has been described previously who presented at age 13 with vitamin B₁₂ (B₁₂) deficiency secondary to a functionally abnormal intrinsic factor (IF). IF has now been isolated from the gastric juice of the patient, his sister, and both parents, who are first cousins, by using affinity chromatography on B₁₂-Sepharose. Patient IF appeared normal in terms of (a) B₁₂ binding, (b) mol wt, (c) total amino acid and carbohydrate composition, and (d) immunodiffusion with rabbit anti-patient and anti-normal IF sera. After adsorption with normal IF, however, anti-patient IF serum precipitated the various IFs as follows: patient IF (> 95%); mother, father, and sister IF (50%); and normal IF (< 10%). Additional adsorption with mother, father, or sister IF completely inhibited the precipitation of patient IF. The association constant determined for patient IF-B₁₂ and human ileal mucosal homogenates ($0.1 \times 10^9 \text{ M}^{-1}$) was 60-fold lower than that determined with normal IF-B₁₂ ($6.0 \times 10^9 \text{ M}^{-1}$). Intermediate amounts of ileal IF-B₁₂ binding were observed with mother, father, and sister IF-B₁₂. These in vitro studies were supported by multiple Schilling tests, performed with a totally gastrectomized volunteer, that gave the following mean urinary excretions of [⁵⁷Co]B₁₂: free B₁₂ (0.5%); + patient gastric juice (2.6%); + mother or father gastric juice (17%); and + normal gastric juice (26%). These studies demonstrate that the patient is homozygous and that the mother, father, and sister are heterozygous for a structurally abnormal IF that has a markedly decreased, but not absent, affinity for ileal IF-B₁₂ receptors. These studies also indicate that the

B₁₂ and ileal binding sites are located on different portions of the IF molecule.

INTRODUCTION

A patient has been described previously (1) who presented at age 13 with glossitis and megaloblastic anemia due to an unusual type of vitamin B₁₂ (B₁₂)¹ malabsorption. Classic pernicious anemia was ruled out on the basis of a histologically normal gastric mucosa, normal gastric acidity, and the absence of serum antibodies against intrinsic factor B₁₂-binding protein (IF). Generalized malabsorption was ruled out by a normal gastrointestinal radiographic examination, normal D-xylose and fat absorption studies, and negative stool examinations for ova and parasites. Isolated congenital absence of IF (2, 3), a congenital selective ileal absorptive defect associated with proteinuria (4, 5), and isolated congenital absence of plasma transcobalamin II (6) were also ruled out since (a) the patient's gastric juice contained normal amounts of a B₁₂-binding protein that was indistinguishable from IF in terms of its immunologic properties, its apparent affinity for B₁₂, and its behavior during standard chromatographic procedures, (b) normal human gastric juice corrected the patient's B₁₂ malabsorption and proteinuria was absent, and (c) the patient's plasma levels of transcobalamin I and II were normal. Additional studies revealed that the patient's gastric juice had a decreased ability to facilitate in vivo B₁₂ absorption by a subject with a total gastrectomy and a decreased ability to facilitate B₁₂ binding to guinea pig ileal homogenates. Gastric juice from both parents, who

This work was presented in part at the Annual Meeting of the American Society of Hematology, Chicago, Ill., 4 December 1973.

Received for publication 17 October 1973 and in revised form 17 December 1973.

¹ Abbreviations used in this paper: B₁₂, vitamin B₁₂; IF, intrinsic factor; vitamin B₁₂-binding protein; pseudo-B₁₂ (adenylyl)-cobamide cyanide.

the first cousins, had an intermediate ability to facilitate *in vivo* B₁₂ absorption.

On the basis of the studies listed above it was postulated that the patient was homozygous for a functionally abnormal IF (1). In order to test this hypothesis, IF has now been isolated from the patient and his mother, father, and sister, and the properties of these preparations have been compared with those of IF isolated from normal individuals.

METHODS

Assay of B₁₂. [⁵⁷Co]B₁₂ (150–200 μCi/μg) and [⁵⁸Co]B₁₂ (3 μCi/μg) were obtained from Amersham/Searle Corp. (Arlington Heights, Ill.) and diluted with nonradioactive crystalline B₁₂ (Sigma Chemical Co., St. Louis, Mo.). Items containing [⁵⁷Co]B₁₂ and [⁵⁸Co]B₁₂ were assayed by measuring radioactivity in a Packard gamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Solutions of crystalline B₁₂ and (α-adenyl)-cobamide cyanide (pseudo-B₁₂) (obtained from Dr. Joseph Pfaffner of Wayne State University) dissolved in water were assayed spectrophotometrically as described previously (7, 8).

B₁₂ binding studies. B₁₂ binding ability was assayed in 0.1 M potassium phosphate, pH 7.5, by a modification (8) of the charcoal adsorption technique of Gottlieb, Lau, Wasserman, and Herbert (9). Anti-IF blocking antibody was obtained from the serum of a pernicious anemia patient as described previously (8). Equilibrium dialysis experiments and experiments designed to measure the ability of [⁵⁷Co]-B₁₂ to displace nonradioactive B₁₂ and pseudo-B₁₂ from IF were also performed as described previously (8).

Purification of IF from gastric juice. Histalog-stimulated gastric juice was collected on ice by nasogastric suction and stored at –20°C after depepsinization (8, 10). IF was isolated by using affinity chromatography on B₁₂-Sephadex as the sole purification step. This step was performed as described previously (8) except that IF was eluted from B₁₂-Sephadex with 5.0 M guanidine-HCl instead of 7.5 M guanidine-HCl. This modification (11) is advantageous since it separates IF from the R-type B₁₂-binding protein that is found in gastric juice in variable amounts. The R-type protein is not eluted with 5.0 M guanidine-HCl but can be eluted subsequently after 16 h of incubation with 7.5 M guanidine-HCl.

Saturation of B₁₂-binding proteins with [⁵⁷Co]B₁₂. A threefold excess (based on B₁₂ binding activity) of [⁵⁷Co]B₁₂ was added to individual B₁₂-binding proteins (1–3 μg protein/ml) in 5.0 M guanidine-HCl containing 0.1 M potassium phosphate, pH 7.5. Proteins were dialyzed subsequently for 72 h at 4°C against 2,000 volumes of 0.05 M potassium phosphate, pH 7.5, containing 0.75 M NaCl with dialysate changes at 24 and 48 h. More than 99% of unbound B₁₂ is removed under these conditions. Protein preparations devoid of B₁₂ were prepared in the same manner except that B₁₂ was not added before dialysis.

Immunoprecipitation of IF-B₁₂ in 30% (NH₄)₂SO₄. Test tubes containing 0.2 ml of serum, consisting of varying amounts of control and anti-IF sera, and 0.1 ml of 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl containing 500 pg of [⁵⁷Co]B₁₂ bound to IF were incubated at 22°C for 30 min. The tubes were then placed in an ice bath and 0.25 ml of cold, saturated (NH₄)₂SO₄ was added. After standing for an additional 30 min, the tubes were centrifuged at 10,000 g for 15 min and 0.2 ml of the supernatant solu-

tion was removed and assayed for [⁵⁷Co]B₁₂. Less than 10% of IF-B₁₂ is precipitated under these conditions in the presence of 0.2 ml of human or rabbit control sera.

Adsorption of rabbit anti-patient IF serum with normal IF. Rabbit anti-patient IF serum (10 ml) was applied to a column (0.9 cm diameter and 5.0 cm tall) of B₁₂-Sephadex containing 1.66 mg (i.e., 50 μg of B₁₂ binding ability) of bound normal IF. The column was equilibrated and eluted at 20°C with 0.01 M potassium phosphate, pH 7.5, 0.14 M NaCl. The flow rate was 10 ml/h and 2-ml fractions were collected. Fractions 3 and 4 contained significantly greater antibody reactivity against patient IF-B₁₂ than against normal IF-B₁₂, as judged by immunoprecipitation assays (see above), and these two fractions were pooled. Fractions 1 and 2 consisted essentially of buffer while fractions 5–7 contained significant amounts of antibody with specificity for both patient and normal IF. Pooled fractions 3 and 4 were adsorbed further by the addition of nonradioactive normal IF-B₁₂ (300 ng B₁₂/ml of serum) 30 min before being used for detailed immunoprecipitation studies.

Assay of IF-B₁₂ binding to intestinal mucosal homogenates. The binding of IF-B₁₂ to intestinal mucosal homogenates (12) was assayed by using a Millipore filter technique (13) as modified and described previously (14). Assays were performed in Krebs-Ringer phosphate, pH 7.5, and in a modified medium in which calcium and magnesium were replaced with 0.001 M Na₂-EDTA. The difference between IF-B₁₂ bound to intestinal mucosal homogenates in these two media was termed the "EDTA-inhibitable" fraction. The validity of using EDTA-inhibitable B₁₂ binding as a measure of specific IF-B₁₂ binding to intestinal mucosal IF-B₁₂ binding sites has been demonstrated previously (14).

Schilling tests. Schilling tests were performed as described previously (1). Informed consent was obtained from the subject who had previously undergone total gastrectomy for carcinoma of the stomach. The amount of B₁₂ ingested contained 0.5 μCi of [⁵⁷Co]B₁₂/test.

Other methods. Polyacrylamide disk gel electrophoresis (8), sodium dodecyl sulfate polyacrylamide gel electrophoresis (8), absorption spectra (8), immunization of rabbits (15), immunodiffusion (15), amino acid and carbohydrate analyses (8), and molecular weight estimation by gel filtration (14) were all performed as described previously. Human plasma transcobalamin II (16), human milk B₁₂-binding protein (15), human saliva B₁₂-binding protein (15), and human granulocyte B₁₂-binding protein (17) were isolated as described previously.

RESULTS

Purification of IF. The purification of IF from gastric juice obtained from normal subjects, the patient, and the patient's father, mother, and sister are summarized in Table I. The data presented demonstrate that all of the gastric juice preparations were normal in terms of total B₁₂ binding ability and in terms of the amount and percent of B₁₂ binding ability attributable to IF (18). The B₁₂-binding protein present in the various gastric juice preparations behaved normally during adsorption to B₁₂-Sephadex and during the subsequent washing and elution steps. The yield of IF obtained ranged from 59 to 87%. All of the B₁₂ binding ability present in the final



FIGURE 1 Polyacrylamide disk gel electrophoresis of the various IF preparations. Each sample contained 25 μ g of protein. Protein samples devoid of B_{12} were renatured from guanidine by dialysis against H_2O for 24 h at 4°C. Protein samples saturated with B_{12} were renatured as described above except that excess B_{12} was added before dialysis. N, normal IF; P, patient IF; F, father IF; M, mother IF; S, sister IF; N + B_{12} , normal IF- B_{12} ; P + B_{12} , patient IF- B_{12} .

preparations could be inhibited with anti-IF-blocking antibody obtained from the serum of a patient with pernicious anemia. All of the final preparations were homogeneous based on disk gel electrophoresis (see below).

Disk gel electrophoresis. When 25 μ g of patient IF, normal IF, mother IF, father IF, and sister IF were subjected to polyacrylamide disk gel electrophoresis in the absence of B_{12} , single protein bands with the same mobility were observed in each case, as shown in Fig. 1. Multiple protein bands were observed, however, when patient IF and normal IF were studied as their B_{12} complexes. This phenomenon has been observed previously with normal IF and results from the fact that human IF aggregates in the presence of B_{12} under certain conditions and exists as a mixture of monomers, dimers, and higher molecular weight oligomers (8). Consistent and reproducible differences in oligomer formation between patient and normal IF- B_{12} were not observed.

Immunologic studies. Single precipitation lines with a pattern of identity were observed with purified patient IF- B_{12} , normal IF- B_{12} , father IF- B_{12} , mother IF- B_{12} , sister IF- B_{12} , and an equal mixture of patient and normal IF- B_{12} when these preparations were subjected to immunodiffusion against both rabbit anti-patient IF (Fig. 2A) and rabbit anti-normal IF (Fig. 2B) sera. Rabbit anti-patient IF serum did not give precipitation lines with human transcobalamin II nor with B_{12} -binding proteins isolated from human milk, saliva, and granulocytes. Patient IF- B_{12} was not precipitated on immunodiffusion by rabbit anti-human transcobalamin II, anti-human milk B_{12} -binding protein, anti-human saliva B_{12} -binding protein, nor control sera (data not shown).

Patient IF- B_{12} and normal IF- B_{12} were precipitated in an equivalent manner (data not presented) when quantitative immunoprecipitation studies were performed with varying amounts of serum, obtained from a pernicious anemia patient, that contained anti-IF binding antibodies. Evidence of extensive, but not necessarily complete, cross-reactivity between patient IF- B_{12} and normal IF- B_{12} was obtained when similar studies were

TABLE I
Summary of Purification of Intrinsic Factor by Affinity Chromatography

| Subject | Gastric juice | | | 5.0 M guanidine-HCl eluate from B_{12} -Sepharose | | | |
|----------|---------------|--------------------------|-------|---|--------------------------|------|-------|
| | Volume | B_{12} binding ability | | Volume | B_{12} binding ability | | Yield |
| | ml | μ g | % IF* | ml | μ g | % IF | % |
| Normals† | 6,580 | 491 | 92 | 64.5 | 358 | 100 | 79 |
| Patient‡ | 1,600 | 125 | 88 | 40.0 | 96.0 | 100 | 87 |
| Father | 257 | 24.8 | 96 | 3.7 | 19.6 | 100 | 82 |
| Mother | 122 | 6.2 | 75 | 4.1 | 3.2 | 100 | 69 |
| Sister | 156 | 9.0 | 88 | 4.0 | 4.7 | 100 | 59 |

* Based on % inhibition of B_{12} binding ability observed with pernicious anemia blocking antibody.

† Pooled gastric juice obtained from 23 different individuals.

‡ Pooled gastric juice from 10 different collections.

TABLE II
Adsorption of Rabbit Anti-Patient IF Serum with
Various IF-B₁₂ Preparations

| Nonradio- active item present in 50-fold excess* | Precipitation of IF-B ₁₂ (0.5 nM [⁵⁷ Co] B ₁₂) by 3 μl of rabbit anti-patient IF serum previously adsorbed with normal IF-B ₁₂ | | | | |
|--|--|-------------------------------|------------------------------|------------------------------|------------------------------|
| | Normal IF-B ₁₂ | Patient IF-B ₁₂ | Father IF-B ₁₂ | Mother IF-B ₁₂ | Sister IF-B ₁₂ |
| | % | % | % | % | % |
| None | 3 | 72 | 43 | 44 | 44 |
| Normal IF-B ₁₂ | 0 | 66 | 36 | 38 | 38 |
| Patient IF-B ₁₂ | 0 | 0 | 0 | 1 | 0 |
| Father IF-B ₁₂ | 0 | 1 | 0 | 2 | 0 |
| Mother IF-B ₁₂ | 0 | 1 | 0 | 6 | 7 |
| Sister IF-B ₁₂ | 0 | 9 | 2 | 8 | 1 |

* Nonradioactive items were added to standard immunoprecipitation assays 30 min before the addition of IF-[⁵⁷Co]B₁₂.

performed with rabbit anti-patient IF and anti-normal IF sera. The results of the studies employing rabbit anti-patient IF serum are presented in Fig. 3A and suggest that this serum is slightly more reactive against patient IF-B₁₂ than against normal IF-B₁₂. A similar, but opposite, difference was observed with rabbit anti-normal IF serum (data not presented). Mother IF-B₁₂, father IF-B₁₂, and sister IF-B₁₂ were also completely precipitated by the two rabbit antisera although they could not be distinguished with confidence from patient IF-B₁₂ nor from normal IF-B₁₂.

Immunologic differences among the various IF-B₁₂ preparations were demonstrated clearly, however, when immunoprecipitation experiments were performed with anti-patient IF serum that had been adsorbed with normal IF-B₁₂. The results are presented in Fig. 3B and

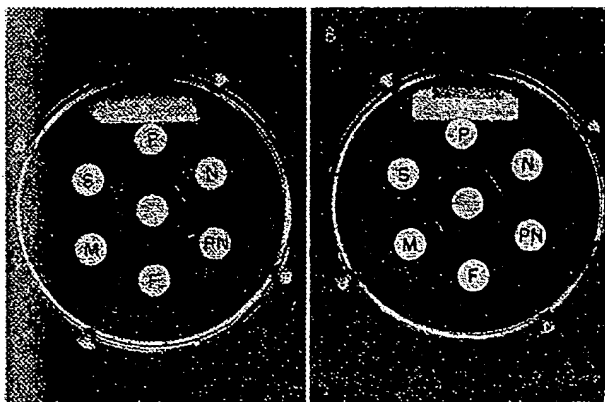


FIGURE 2 Immunodiffusion with rabbit anti-patient IF and anti-normal IF sera. The center wells contained 25 μl of (A) anti-patient IF serum and (B) anti-normal IF serum. The outer wells contained 20 μl of the various IF-B₁₂ preparations (5 μg B₁₂/ml). P, patient IF-B₁₂; N, normal IF-B₁₂; PN, 1/2 patient IF-B₁₂ + 1/2 normal IF-B₁₂; F, father IF-B₁₂; M, mother IF-B₁₂; S, sister IF-B₁₂.

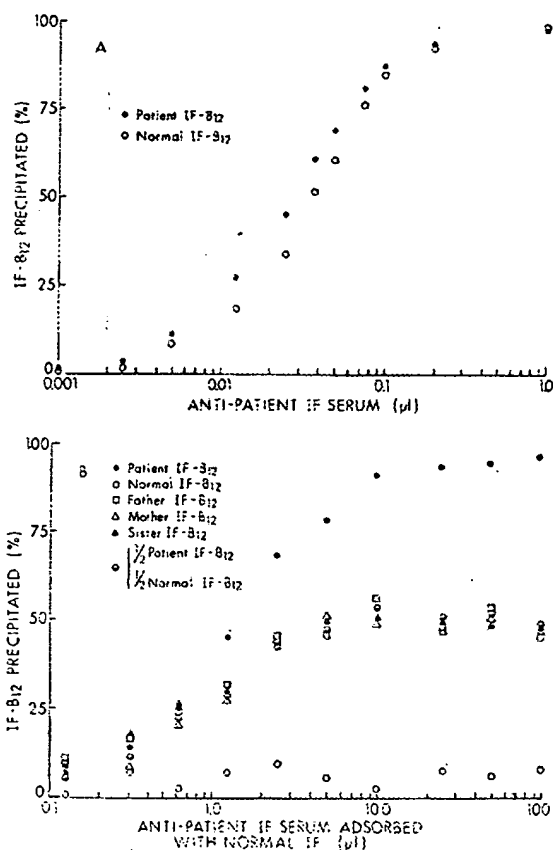


FIGURE 3 Immunoprecipitation assays with rabbit anti-patient IF sera and the various IF-B₁₂ preparations. (A) Whole anti-patient IF serum; (B) anti-patient IF serum after adsorption with normal IF-B₁₂.

reveal that the adsorbed anti-patient IF serum was still capable of precipitating patient IF-B₁₂ completely, although its ability to precipitate normal IF-B₁₂ was now negligible. Mother IF-B₁₂, father IF-B₁₂, sister IF-B₁₂, and an equal mixture of patient and normal IF-B₁₂ were precipitated approximately 50% by the adsorbed anti-patient IF serum.

The data presented in Fig. 3B demonstrate that mother IF-B₁₂, father IF-B₁₂, and sister IF-B₁₂ are composed of equal mixtures of normal IF-B₁₂ and an immunologically distinct IF-B₁₂ but the data do not enable one to determine whether these preparations contain the same immunologically distinct IF-B₁₂. In order to answer this question additional immunoprecipitation experiments were performed in which the anti-patient IF serum that had been adsorbed with normal IF-B₁₂ was adsorbed further with the various IF-B₁₂ preparations. The results are presented in Table II and reveal that patient IF-B₁₂ and the immunologically distinct portions of mother, father, and sister IF-B₁₂ all compete for the same antibodies. This observation indicates that patient IF-B₁₂

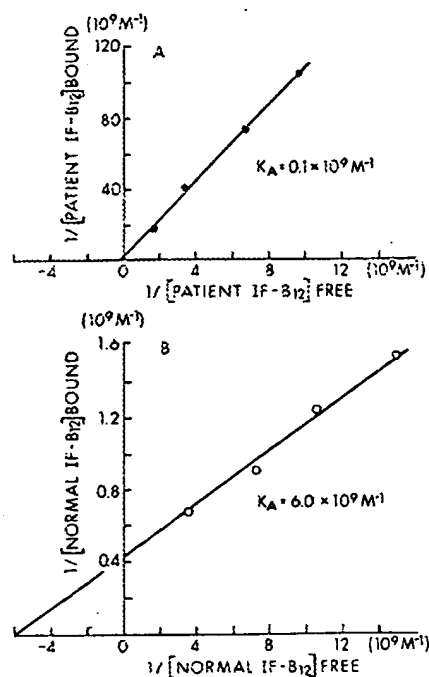


FIGURE 4 Double reciprocal plots of EDTA-inhibitable IF-B₁₂ binding to human ileal mucosal homogenate vs. IF-B₁₂ concentration. (A) patient IF-B₁₂; (B) normal IF-B₁₂.

consists of a single immunologically distinct species and that mother, father, and sister IF-B₁₂ consist of equal mixtures of the same immunologically distinct IF-B₁₂ and normal IF-B₁₂.

A comparison of Figs. 3A and 3B reveals that the potency of the adsorbed anti-patient IF-B₁₂ serum is approximately 2 orders of magnitude less than that of

TABLE III
EDTA-Inhibitable Binding of IF-B₁₂ to Human
Ileal Mucosal Homogenates

| Item | Amount present during incubation | B ₁₂ bound to ileal homogenate* | |
|---|----------------------------------|--|-------|
| | | Range | Mean |
| | pg B ₁₂ /ml | pg | |
| Normal IF-B ₁₂ | 200 | 8.80-9.33 | 8.99 |
| Patient IF-B ₁₂ | 200 | 0.05-0.37 | 0.20† |
| Father IF-B ₁₂ | 200 | 5.39-5.77 | 5.52 |
| Mother IF-B ₁₂ | 200 | 5.57-6.65 | 5.92 |
| Sister IF-B ₁₂ | 200 | 5.58-6.23 | 5.89 |
| ½ Normal IF-B ₁₂ , ½ patient IF-B ₁₂ | 200 | 5.17-5.99 | 5.44 |
| Normal IF-B ₁₂ | 100 | 5.60-6.37 | 5.99 |

* Assays were performed in triplicate except for assays with patient IF-B₁₂ which were performed in quadruplicate.

† Significantly different from zero with $P < 0.05$.

TABLE IV
Schilling Tests Performed with a Single Subject
with a Total Gastrectomy

| Date | [Co ⁵⁷]B ₁₂ administered | Gastric juice administered* | 48 h urinary excretion of [Co ⁵⁷]B ₁₂ |
|----------|--|-----------------------------|--|
| | μg | | % |
| 10/14/70 | 2.0 | normal | 24.1 |
| 10/21/70 | 2.0 | none | 0.8 |
| 11/9/70 | 2.0 | patient | 2.8 |
| 3/25/71 | 1.0 | normal | 26.3 |
| 11/18/70 | 1.0 | ½ normal, ½ patient | 17.7 |
| 3/15/71 | 1.0 | father | 17.5 |
| 3/18/71 | 1.0 | mother | 17.9 |
| 10/5/70 | 0.5 | normal | 26.6 |
| 6/7/73 | 0.5 | normal | 26.1 |
| 10/21/70 | 0.5 | none | 0.3 |
| 6/14/73 | 0.5 | none | 0.8 |
| 6/21/73 | 0.5 | none | 0.5 |
| 7/4/73 | 0.5 | none | 0.5 |
| 10/19/70 | 0.5 | patient | 3.4 |
| 6/11/73 | 0.5 | patient | 1.5 |
| 6/18/73 | 0.5 | patient | 3.4 |
| 6/30/73 | 0.5 | patient | 2.1 |
| Means | 0.5 μg [Co ⁵⁷]B ₁₂ without gastric juice | 0.5% | † |
| | 0.5 μg [Co ⁵⁷]B ₁₂ with patient gastric juice | 2.6% | † |

* The amount of gastric juice administered was such that all of the B₁₂ administered could be bound to IF. Gastric juice and [Co⁵⁷]B₁₂ were incubated together for 15 min before administration.

† These two mean values differ significantly with $P < 0.01$.

the unadsorbed anti-patient IF-B₁₂ serum in terms of its ability to precipitate patient IF-B₁₂. This observation, together with the fact that the protein concentrations of the two sera were approximately the same, suggests that only several percent of the anti-patient IF-B₁₂ antibodies present in the whole anti-serum are specific for patient IF-B₁₂ as opposed to normal IF-B₁₂.

Ileal binding studies. The amounts of EDTA-inhibitable IF-B₁₂ binding to human ileal mucosal homogenates observed with the various IF-B₁₂ preparations are presented in Table III. A statistically significant amount

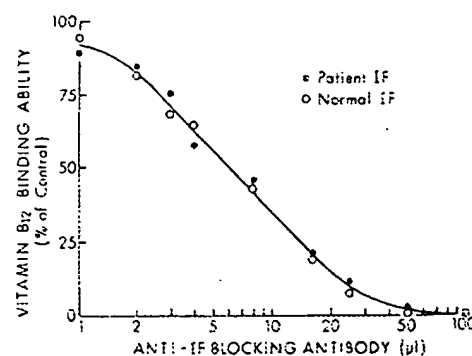


FIGURE 5 Inhibition of the B₁₂ binding abilities (500 pg B₁₂) of purified patient IF and normal IF by use of anti-IF blocking antibodies obtained from the serum of a patient with pernicious anemia.

of such binding was observed with patient IF-B₁₂ but the amount observed at a B₁₂ concentration of 200 pg/ml was less than 3% of that observed with normal IF-B₁₂. Intermediate values (61–66% of normal) were observed with mother IF-B₁₂, father IF-B₁₂, sister IF-B₁₂, and an equal mixture of patient and normal IF-B₁₂. Similar results were observed with monkey, hog, dog, and guinea pig ileal mucosal homogenates.

The amounts of EDTA-inhibitable ileal IF-B₁₂ binding observed at varying concentrations of IF-B₁₂ were used to calculate values for the association constants for patient and normal IF-B₁₂ and human ileal homogenates as shown in Fig. 4. Values of $0.1 \times 10^6 \text{ M}^{-1}$ and $6.0 \times 10^6 \text{ M}^{-1}$ were obtained with patient IF-B₁₂ and normal IF-B₁₂, respectively. Additional human ileal binding studies have been performed and reveal that the markedly low values for B₁₂ binding observed with patient IF-B₁₂ are not increased when the calcium and/or magnesium concentrations in the incubation medium are increased between 10 and 100-fold or when the pH is varied between 6.5 and 9.5. Patient IF does not appear to be subjected to extensive proteolysis during incubation with human ileal homogenates since after a 3-h incubation more than 90% of unbound patient IF-B₁₂ is precipitated with anti-IF serum and also elutes from Sephadex G-150 with an apparent molecular weight of approximately 60,000, i.e., the apparent molecular weight of native patient IF-B₁₂. Similar results have been reported previously with normal IF-B₁₂ (14).

Schilling tests. The results of multiple Schilling tests performed with a single individual with a total gastrectomy are presented in Table IV. These studies demonstrate that the patient's gastric juice is able to facilitate B₁₂ absorption in vivo although the amount of facilitation is only approximately 10% of that observed with normal human gastric juice. Intermediate levels of facilitation, i.e., 60–70% of that observed with normal human gastric juice, were observed with gastric juice from the patient's father and mother. A similar intermediate value was ob-

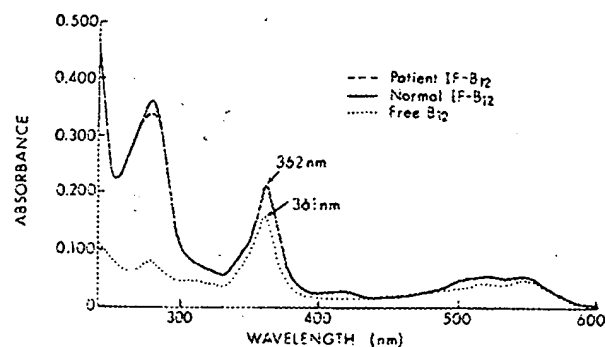


FIGURE 6 Absorption spectra of equal concentrations (7.25 µg B₁₂/ml) of patient IF-B₁₂, normal IF-B₁₂, and free B₁₂. Spectra were obtained at 22°C in 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl.

served with an equal mixture of patient and normal gastric juice.

B₁₂ binding studies. The ability of patient IF to bind B₁₂ appeared normal when measured by a variety of techniques. The data presented in Fig. 5 reveal that the B₁₂ binding abilities of patient IF and normal IF are inhibited in an equivalent manner by anti-IF blocking antibody obtained from the serum of a patient with pernicious anemia. The data presented in Table V indicate that purified patient IF and normal IF bind B₁₂ at the same rate at 4°C. The rate observed was indistinguishable from the rate observed when crude patient and normal gastric juice were used as the source of IF (data not presented). Pseudo-B₁₂ is displaced from both proteins at a faster rate than is native B₁₂. Similar displacement rates were observed with both patient IF and normal IF. Studies of this kind were also performed at 37°C (data not presented). The rates of B₁₂ binding and displacement were significantly faster at 37°C than at 4°C but no differences between patient and normal IF were observed. The association constants for the two proteins and B₁₂ were determined by equilibrium dialysis in

TABLE V
Displacement of B₁₂ and Pseudo-B₁₂ with [⁵⁷Co]B₁₂ at 4°C

| Item | Nonradioactive item present during 30-min preincubation | [⁵⁷ Co]B ₁₂ bound at different time periods after the addition of 1,000 pg | | | | | | |
|------------|---|---|---------|---------|---------|--------|--------|---------|
| | | 0.5 min | 1.0 min | 2.0 min | 5.0 min | 10 min | 30 min | 120 min |
| | | pg | | pg | | pg | | % |
| Normal IF | None | 126 | 194 | 310 | 421 | 463 | 499 | 100.0 |
| Normal IF | 1,500 pg pseudo-B ₁₂ | 82 | 135 | 200 | 329 | 427 | 482 | 100.8 |
| Normal IF | 1,500 pg B ₁₂ | 0 | 0 | 0 | 2 | 1 | 3 | 1.6 |
| Patient IF | None | 146 | 228 | 320 | 448 | 487 | 508 | 100.0 |
| Patient IF | 1,500 pg pseudo-B ₁₂ | 86 | 129 | 210 | 370 | 422 | 500 | 96.9 |
| Patient IF | 1,500 pg B ₁₂ | 0 | 0 | 1 | 0 | 2 | 4 | 1.7 |

TABLE VI
Amino Acid and Carbohydrate Composition

| Item | Normal IF | | Patient IF |
|--------------------------|---------------------------|---------------------------|---------------------------|
| | 1st preparation | Present preparation | |
| | mol/mol B ₁₂ * | mol/mol B ₁₂ † | mol/mol B ₁₂ † |
| Amino acid | | | |
| Lysine | 20 | 19 | 19 |
| Histidine | 5 | 4 | 5 |
| Arginine | 5 | 6 | 6 |
| Aspartic | 38 | 38 | 38 |
| Threonine | 24 | 24 | 26 |
| Serine | 30 | 30 | 31 |
| Glutamic | 35 | 36 | 36 |
| Proline | 22 | 21 | 24 |
| Glycine | 20 | 20 | 22 |
| Alanine | 23 | 23 | 22 |
| Valine | 22 | 20 | 23 |
| Isoleucine | 22 | 20 | 21 |
| Leucine | 34 | 33 | 34 |
| Tyrosine | 9 | 9 | 9 |
| Phenylalanine | 10 | 10 | 10 |
| Methionine | 10 | 10 | 10 |
| Half-cystine | 6 | 6 | 6 |
| Tryptophan | 6 | 5 | 5 |
| Total | 342 | 334 | 347 |
| (Molecular weight) | (37,500) | (36,600) | (37,900) |
| Carbohydrate | | | |
| Fucose | 7 | 6 | 8 |
| Galactose | 6 | 3 | 3 |
| Mannose | 12 | 13 | 12 |
| Galactosamine | 3 | 5 | 5 |
| Glucosamine | 6 | 5 | 5 |
| Sialic Acid | 3 | 2 | 2 |
| Total | 37 | 34 | 35 |
| (Molecular weight) | (6,600) | (6,100) | (6,200) |
| Total number of residues | 379 | 368 | 382 |
| (Total molecular weight) | (44,100) | (42,700) | (44,100) |
| (% carbohydrate) | (15.0%) | (14.3%) | (14.1%) |

* Average of duplicate analyses. These values have been published previously (8).

† Single determinations.

0.1 M potassium phosphate, pH 7.5, at 4°C. Values of $1.3\text{--}1.6 \times 10^6 \text{ M}^{-1}$ were obtained for both patient and normal IF.

The spectra of equal concentrations of patient IF-B₁₂, normal IF-B₁₂, and unbound B₁₂ are presented in Fig. 6. When B₁₂ is bound to either patient or normal IF the spectral maximum for B₁₂ shifts from 361 nm to 362 nm and the absolute absorbance at 361 nm increases by 30%. Previous studies (8) employing normal IF have demonstrated that the increase in absolute absorbance at 361 nm is due to the interaction of IF and B₁₂ rather than to a possible isotope effect that could result in erroneously high values being determined for the concentration of IF-B₁₂. The absorbance of the patient IF-B₁₂ complex at 280 nm was slightly lower than that of normal IF-B₁₂ and resulted in an A_{280}/A_{361} value of 1.62 for patient IF-B₁₂ which is lower than the value of 1.72

that was observed with normal IF-B₁₂. A value of 1.68 was obtained with a different preparation of normal IF-B₁₂ (8). The significance, if any, of these differences is unknown.

Molecular weight studies. The molecular weight of patient IF appeared normal when estimated by a variety of techniques. The results of amino acid and carbohydrate analyses are presented in Table VI. By using the molecular weights of the individual amino acids and carbohydrates, it was determined that patient IF contains 44,100 g of amino acid and carbohydrate/mol of bound B₁₂. This value is not significantly different from the values of 44,100 and 42,700 g obtained with two separate preparations of normal IF. All three of these values are in good agreement with the molecular weight values of 44,000–48,000 that we have obtained previously (8) for normal IF using sedimentation equilibrium ultracentrifugation. The data presented in Table VI also fail to reveal any significant differences in the amino acid and carbohydrate composition of patient and normal IF. Minor differences can not be relied on or necessarily detected, on the basis of a small number of analyses.

When patient IF and normal IF were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence of 1% 2-mercaptoethanol single protein bands with apparent molecular weights of 55,000 were observed in both cases.²

In studies employing gastric juice and gel filtration other investigators (23–25) have observed that human IF-B₁₂ has a smaller apparent molecular weight than human IF devoid of B₁₂. These studies have suggested that the conformation of human IF changes to a more compact form when B₁₂ is bound to it. This observation and interpretation are supported by the gel filtration experiments performed with homogeneous human IF that are presented in Fig. 7. These experiments demonstrate that both patient and normal IF devoid of B₁₂ elute from Sephadex G-150 with apparent molecular weights of 70,000 while both patient IF-B₁₂ and normal IF-B₁₂ elute with apparent molecular weights of 61,000.⁴

When patient IF and normal IF were saturated with [⁶⁰Co]B₁₂ for 72 h before being subjected to gel filtration multiple peaks of radioactivity were observed (data not presented) indicating that both proteins had aggregated as has been observed previously with normal IF-B₁₂ (8). No differences between patient IF-B₁₂ and normal IF-B₁₂ were observed in terms of the amount or nature of this kind of aggregation.

⁴ The molecular weight estimates obtained for human IF with sodium dodecyl sulfate gel electrophoresis and gel filtration appear falsely elevated when compared with the values obtained by amino acid and carbohydrate analyses and sedimentation equilibrium ultracentrifugation. Discrepancies of this type have been noted previously with other glycoproteins (15, 17, 19–22).

DISCUSSION

From the existing knowledge of the process of IF-mediated B_{12} absorption, it appears likely that mutations in the structural gene for IF could cause B_{12} malabsorption by a variety of mechanisms. Such mutations might, for example, result in a decrease in or absence of synthesis of IF by gastric parietal cells or in a decrease in secretion of IF into the gastric juice. Other mutations might result in IF molecules that have a decrease in or absence of affinity for B_{12} or an increase in susceptibility to proteolytic enzymes such as pepsin, trypsin, chymotrypsin, etc. Still other mutations might result in IF molecules that have as their IF- B_{12} complexes a decrease in or absence of affinity for ileal IF- B_{12} receptors. A structurally abnormal IF could also interfere with the subsequent ileal phase of B_{12} absorption. This phase is poorly understood although at some point B_{12} must be released from IF since IF does not appear to enter the portal blood (26, 27).

The patient described in this report represents the first documented case of B_{12} malabsorption secondary to a structurally abnormal IF. The immunologic studies performed with rabbit anti-patient IF serum demonstrate that patient IF contains at least one, though not many, antigenic determinant that is not present on normal IF. The studies performed with rabbit anti-normal IF serum suggest that the reverse is also true. These observations, together with the fact that patient IF and normal IF have very similar molecular weights and amino acid and carbohydrate compositions, are compatible with a single amino acid substitution, although this has not been proved.

Schilling tests, ileal homogenate binding studies, and immunologic studies all indicate that the patient's mother, father, and sister have gastric juices that contain equal mixtures of normal IF and a structurally abnormal IF. These studies thus demonstrate that mutations in the structural gene for IF are inherited as autosomal recessive traits. The antibody adsorption studies also indicate that the mother, father, and sister all possess the same structurally abnormal IF, an observation that is not unexpected since the parents are first cousins. These studies also indicate that the patient is homozygous for a single structurally abnormal IF.

The ileal homogenate binding studies reported here demonstrate that patient IF- B_{12} has a decreased affinity for ileal IF- B_{12} receptors and indicate that this functional abnormality is responsible for the patient's B_{12} malabsorption. The finding that this affinity is decreased rather than absent is supported by the results of multiple Schilling tests that demonstrate that the patient's gastric juice has a decreased but not absent ability to facilitate B_{12} adsorption *in vivo*. This small amount of activity may account for the fact that the patient did

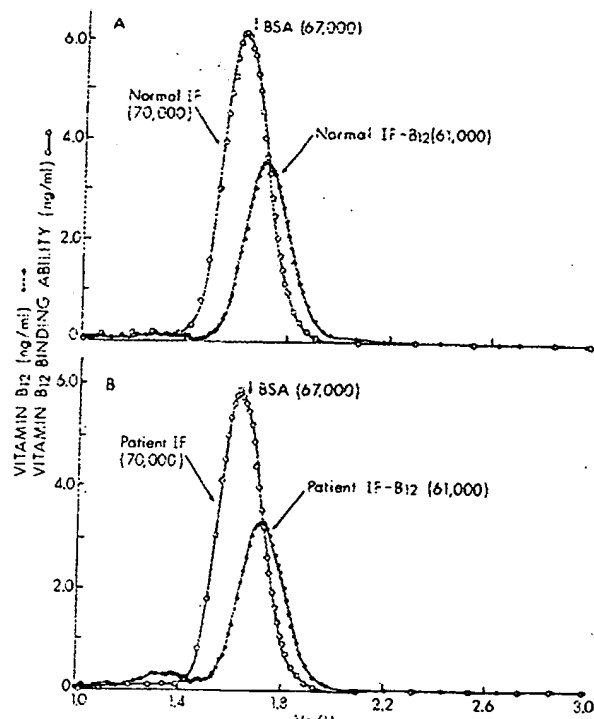


FIGURE 7 Gel filtration experiments. Protein samples were prepared in 1 ml of 0.05 M potassium phosphate, pH 7.5, 0.75 M NaCl and contained, in order of addition, IF (240 ng of B_{12} binding ability), bovine serum albumin (20 mg), blue dextran (2.5 mg), and [^{57}Co] B_{12} (83 ng). After standing for 1 h at 4°C, samples were applied to a 2.0 × 90 cm column of Sephadex G-150, equilibrated with the same buffer, and 1-ml fractions were collected. Fractions were assayed for blue dextran (A_{660}), bovine serum albumin (A_{280}), IF- B_{12} ([^{57}Co] B_{12}), and free IF (B_{12} binding ability). B_{12} binding assays were performed with [^{57}Co] B_{12} at 4°C; negligible exchange of B_{12} occurs under these conditions (see Table V). (A) Normal IF; (B) patient IF.

not develop clinical B_{12} deficiency until age 13, an age that is considerably greater than that (< 5 years of age) observed with patients with a complete lack of IF-facilitated B_{12} absorption (2-5).

A number of children have been reported in the literature (2, 3, 28-43) as cases of B_{12} malabsorption secondary to congenital absence of IF. The diagnostic criteria have consisted in most cases of presentation with B_{12} deficiency before age 5, Schilling test results that indicate a lack of IF activity, and the demonstration of a histologically normal gastric mucosa and normal gastric acidity. From the discussion above it is clear that some, if not all, of these patients may actually represent examples of B_{12} malabsorption due to structurally abnormal IFs. In some of the cases just mentioned (3, 38-42) samples of gastric juice were actually shown to lack IF by studies based on assays employing either the ability of anti-IF blocking antibodies to block

[^{57}Co] B_{12} binding in the patient's gastric juice or the ability of anti-IF binding antibodies to bind to protein- B_{12} complexes formed after the addition of [^{57}Co] B_{12} to the patient's gastric juice. The fact that no IF was observed with these assays rules out the presence of a structurally abnormal IF of the kind described in this report but does not rule out the presence of a structurally abnormal IF that has a decrease in or absence of affinity for B_{12} .

In previous studies (14) it was observed that 100-fold excesses of free B_{12} or purified human IF devoid of B_{12} did not cause detectable inhibition of human IF- B_{12} binding to human ileal mucosal homogenates. These observations indicate that free B_{12} and human IF devoid of B_{12} have little if any affinity for human ileal IF- B_{12} receptors and suggest that B_{12} binding to IF results in important conformational changes in the portion of the B_{12} and/or IF molecule that interacts with the ileal IF- B_{12} receptor. The studies performed with the mutant IF reported here make it extremely unlikely, however, that the ileal IF- B_{12} receptor interacts only with the conformationally altered B_{12} molecule since the mutant IF- B_{12} complex binds poorly to the ileal IF- B_{12} receptor despite the fact that mutant IF binds B_{12} normally as judged by studies employing equilibrium dialysis, adsorption to B_{12} -Sephadex, interaction with anti-IF blocking antibodies, absorption spectra, and measurements of B_{12} binding rates and the rates of displacement of B_{12} and pseudo- B_{12} . It thus appears that the ileal IF- B_{12} receptor interacts with a portion of the IF molecule that is distinct from the B_{12} binding site although additional interaction with the B_{12} molecule is not ruled out.

ACKNOWLEDGMENTS

This investigation was supported by a grant (MA 4764) from the Medical Research Council of Canada and by a grant (AM 16668) from the National Institutes of Health, U. S. Public Health Service.

REFERENCES

- Katz, M., S. K. Lee, and B. A. Cooper. 1972. Vitamin B_{12} malabsorption due to a biologically inert intrinsic factor. *N. Engl. J. Med.* 287: 425.
- Spurling, C. L., M. S. Sacks, and R. M. Jiji. 1964. Juvenile pernicious anemia. *N. Engl. J. Med.* 271: 995.
- McIntyre, O. R., L. W. Sullivan, G. H. Jeffries, and R. H. Silver. 1965. Pernicious anemia in childhood. *N. Engl. J. Med.* 272: 981.
- Imerslund, O. 1960. Idiopathic chronic megaloblastic anemia in children. *Acta Paediatr. Scand. Suppl.* 119: 1.
- Grasbeck, R., R. Gordon, I. Kantero, and B. Kuhlback. 1960. Selective vitamin B_{12} malabsorption and proteinuria in young people: a syndrome. *Acta Med. Scand.* 167: 289.
- Hakami, N., P. E. Neiman, G. P. Canellos, and J. Lazerson. 1971. Neonatal megaloblastic anemia due to inherited transcobalamin II deficiency in two siblings. *N. Engl. J. Med.* 285: 1163.
- Allen, R. H., and P. W. Majerus. 1972. Isolation of vitamin B_{12} -binding proteins using affinity chromatography. I. Preparation and properties of vitamin B_{12} -Sephadex. *J. Biol. Chem.* 247: 7695.
- Allen, R. H., and C. S. Mehman. 1973. Isolation of gastric vitamin B_{12} -binding proteins using affinity chromatography. I. Purification and properties of human intrinsic factor. *J. Biol. Chem.* 248: 3660.
- Gottlieb, C., K. S. Lau, L. Wasserman, and V. Herbert. 1965. Rapid charcoal assay for intrinsic factor (IF) gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity. *Blood J. Hematol.* 25: 875.
- Flood, C., and R. West. 1936. Some properties of Castle's intrinsic factor. *Proc. Soc. Exp. Biol. Med.* 34: 542.
- Allen, R. H., R. L. Burger, C. S. Mehman, and P. W. Majerus. 1974. Vitamin B_{12} . *Methods Enzymol.* 34B: in press.
- Sullivan, L. W., V. Herbert, and W. B. Castle. 1963. *In vitro* assay for human intrinsic factor. *J. Clin. Invest.* 42: 1443.
- Ukyo, S., and B. A. Cooper. 1965. Intrinsic factor-like activity in extracts of guinea pig intestine. *Am. J. Physiol.* 208: 9.
- Hooper, D. C., D. H. Alpers, R. L. Burger, C. S. Mehman, and R. H. Allen. 1973. Characterization of ileal vitamin B_{12} binding using homogeneous human and hog intrinsic factors. *J. Clin. Invest.* 52: 3074.
- Burger, R. L., and R. H. Allen. 1974. Purification and properties of human milk and human saliva vitamin B_{12} binding proteins. *J. Biol. Chem.* In press.
- Allen, R. H., and P. W. Majerus. 1972. Isolation of vitamin B_{12} -binding proteins using affinity chromatography. III. Purification and properties of human plasma transcobalamin II. *J. Biol. Chem.* 247: 7709.
- Allen, R. H., and P. W. Majerus. 1972. Isolation of vitamin B_{12} -binding proteins using affinity chromatography. II. Purification and properties of a human granulocyte vitamin B_{12} -binding protein. *J. Biol. Chem.* 247: 7702.
- Irvine, W. J., D. R. Cullen, L. Scarth, and J. D. Simpson. 1968. Intrinsic-factor secretion assessed by direct radioimmunoassay and by total-body counting in patients with achlorhydria and in acid secretors. *Lancet* 2: 184.
- Segrest, J. P., R. L. Jackson, E. P. Andrews, and V. T. Marchesi. 1971. Human erythrocyte membrane glycoprotein: A re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. *Biochem. Biophys. Res. Commun.* 44: 390.
- Bretscher, M. S. 1971. Major human erythrocyte glycoprotein spans the cell membrane. *Nat. New Biol.* 231: 229.
- Andrews, P. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* 96: 595.
- Allen, R. H., and C. S. Mehman. 1973. Isolation of gastric vitamin B_{12} -binding proteins using affinity chromatography. II. Purification and properties of hog intrinsic factor and hog nonintrinsic factor. *J. Biol. Chem.* 248: 3670.
- Grasbeck, R. 1967. Intrinsic factor and the transcobalamins with reflections on the general function and evolution of soluble transport proteins. *Scand. J. Clin. Lab. Invest. Suppl.* 95: 7.

24. Hippe, E. 1970. Changes in stokes radius on binding of B₁₂ to human intrinsic factor and transcobalamins. *Biochim. Biophys. Acta*. 208: 337.
25. Natori, H. 1971. Physicochemical analyses of vitamin B₁₂ binding proteins in sera, gastric juice, and leucocytes. *Tumor Res.* 6: 1.
26. Ardeman, S., I. Chanarin, and V. Berry. 1965. Studies on human gastric intrinsic factor. (Observations on its possible absorption and enterohepatic circulation). *Br. J. Haematol.* 11: 11.
27. Cooper, B. A., and J. J. White. 1968. Absence of intrinsic factor from human portal plasma during ⁵⁷Co-B₁₂ absorption in man. *Br. J. Haematol.* 14: 73.
28. Reisner, E. H., Jr., J. A. Wolff, R. J. McKay, Jr., and E. F. Doyle. 1951. Juvenile pernicious anemia. *Pediatrics*. 8: 88.
29. Molin, D. L., S. J. Baker, and I. Doniach. 1955. Addisonian pernicious anemia without gastric atrophy in young man. *Br. J. Haematol.* 1: 278.
30. Stevenson, T. D., J. A. Little, and L. Langley. 1956. Pernicious anemia in childhood. *N. Engl. J. Med.* 255: 1219.
31. Harris-Jones, J. N., H. T. Swan, and G. R. Tudhope. 1957. Pernicious Anemia without gastric atrophy in the presence of free hydrochloric acid. *Blood J. Hematol.* 12: 461.
32. Leikin, S. L. 1960. Pernicious anemia in childhood. *Pediatrics*. 25: 91.
33. Lambert, H. P., T. A. J. Prankerd, and J. M. Smellie. 1961. Pernicious anemia in childhood: report of two cases in one family and their relationship to aetiology of pernicious anemia. *Q. J. Med.* 30: 71.
34. Clement, D. H., C. A. Nichol, and A. D. Welch. 1961. Case of juvenile pernicious anemia: study of effects of folic acid and vitamin B₁₂. *Blood J. Hematol.* 17: 618.
35. Waters, A. H., and M. E. B. Murphy. 1963. Familial juvenile pernicious anemia: Study of hereditary basis of pernicious anemia. *Br. J. Haematol.* 9: 1.
36. Pearson, H. A., R. Vinson, and R. T. Smith. 1964. Pernicious anemia with neurologic involvement in childhood: report of case with emphasis on changes of folic acid therapy. *J. Pediatr.* 65: 335.
37. Moody, E. A. 1965. Juvenile pernicious anemia. *Northwest Med.* 64: 191.
38. Herbert, V., R. R. Streiff, and L. W. Sullivan. 1964. Notes on vitamin B₁₂ absorption: autoimmunity and pernicious anemia: relation of intrinsic factor to blood group substance. *Medicine (Baltimore)*. 43: 679.
39. Dimson, S. B. 1966. Juvenile pernicious anemia. *Arch. Dis. Child.* 41: 216.
40. Miller, D. R., G. E. Bloom, R. R. Streiff, A. F. LoBuglio, and L. K. Diamond. 1966. Juvenile "Congenital" pernicious anemia. *N. Engl. J. Med.* 275: 978.
41. Lampkin, B. C., and A. M. Mauer. 1967. Congenital pernicious anemia with coexistent transitory intestinal malabsorption of vitamin B₁₂. *Blood J. Hematol.* 30: 495.
42. McNicholl, B., and B. Egan. 1968. Congenital pernicious anemia: effects on growth, brain and absorption of B₁₂. *Pediatrics*. 42: 149.
43. Yun, D. J., H. Lee, G. P. Chun, and K. Y. Lee. 1967. Juvenile pernicious anemia in sisters. *Yonsei Med. J.* 8: 71.